Physiological, biochemical, anthropometric and biomechanical influences on exercise economy in humans

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Running head: Determinants of exercise economy

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Abstract

Inter-individual variation in running and cycling exercise economy (EE) remains unexplained although studied for more than a century. This study is the first to comprehensively evaluate the importance of biochemical, structural, physiological, anthropometric, and biomechanical influences on running and cycling EE within a single study. In 22 healthy males (VO_2max range 45.5 to 72.1 $ml.min^{-1}.kg^{-1}$) no factor related to skeletal muscle structure (% slow twitch fibre content, number of capillaries per fibre), mitochondrial properties (volume density, oxidative capacity, or mitochondrial efficiency) or protein content (UCP3 and MFN2 expression) explained variation in cycling and running EE among subjects. In contrast, biomechanical variables related to vertical displacement correlated well with running EE, but were not significant when taking body weight into account. Thus, running EE and body weight were correlated ($R^2 = 0.94$; $P < 0.001$), but was lower for cycling EE ($R^2 = 0.23$; $P < 0.023$). To separate biomechanical determinants of running EE we contrasted individual running and cycling EE considering that during cycle ergometer exercise the biomechanical influence on EE would be small because of the fixed movement pattern. Differences in cycling and running exercise protocols, e.g., related to biomechanics, play however only a secondary role in determining EE. There was no evidence for an impact of structural or functional skeletal muscle variables on EE. Body weight was the main determinant of EE explaining 94% of variance in running EE, although more than 50% of the variability of cycling EE remains unexplained.

Key words: Biomechanic, locomotion, mitochondria, skeletal muscle.

Abbreviations

Absolute and relative vertical displacement during loading phase (VDL_{ABS} and VDL_{REL}) [cm]

Absolute and relative vertical displacement during pushoff and flightphase (VDP_{ABS} and VDP_{REL}) [cm]

Ankle path (AP) [cm]

Body weight (BW)

Coupling control ratio (CCR)

Cytochrome c oxidase (COX)

Electron transport system (ETS)

Exercise economy (EE)

Carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP)

Initial ankle dorsiflexion (ADF_{INIT}) [°]

Initial hip flexion (HFL_{INIT}) [°]

Initial knee flexion (KFL_{INIT}) [°]

Intermyofibrillar mitochondrial volume density (IMF $Mito_{VD}$)

Fast twitch muscle fibre type IIa (FT IIa)

Fast twitch muscle fibre type IIx (FT IIx)

Fatty acid oxidation (FAO)

Fatty acid oxidative capacity (P_{FAO}) [pmol $O_2 \cdot s^{-1} \cdot$ mg ww^{-1}]

Flight phase (FP) [sec]

Leak control ratio (LCR)

Leak respiratory state in absence of adenylates (L_N) [pmol $O_2 \cdot sec^{-1} \cdot mg\ ww^{-1}$]

Leak respiratory state by inhibition of ATP synthase by oligomycin (L_{OMY}) [pmol $O_2 \cdot sec^{-1} \cdot mg\ ww^{-1}$]

Maximal ankle dorsiflexion during stance phase (ADF_{MAX}) [°]

Maximal ankle plantarflexion during stance phase (DPF_{MAX}) [°]

Maximal hip extension during stance phase (HEX_{MAX}) [°]

Maximal knee extension during stance phase (KEX_{MAX}) [°]

Maximal knee flexion during stance phase (KFL_{MAX}) [°]

Maximal oxidative phosphorylation (OXPHOS) [pmol $O_2 \cdot sec^{-1} \cdot mg\ ww^{-1}$]

Mitochondrial uncoupling protein 3 (UCP3)

Mitochondrial volume density ($Mito_{VD}$)

Mitofusin 2 (MFN2)

N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride (TMPD)

Pulmonary oxygen uptake (VO_2) (units?)

Range of motion of ankle dorsiflexion during stance phase (ADF_{ROM}) [°]

Range of motion of ankle plantarflexion during stance phase (APF_{ROM}) [°]

Range of motion of hip extensions during stance phase (HEX_{ROM}) [°]

Range of motion of knee extensors during stance phase (KEX_{ROM}) [°]

Range of motion of knee flexors during stance phase (KFL_{ROM}) [°]

Slow twitch muscle fibres (ST)

Stance phase (SP) [sec]

Introduction

Exercise economy (EE) represents the ability to convert oxygen to work, e.g. during running and cycling and is expressed as pulmonary oxygen uptake (VO_2) at the mechanical work completed (Gaesser & Brooks 1975). Along with the capacity for O_2 transport to the muscles, EE is a major determinant of exercise performance (Joyner & Coyle 2008; Lundby & Robach 2015). For running EE is typically presented as ml $O_2 \cdot kg^{-1} \cdot km^{-1}$ (although $kcal.kg^{-1}.min^{-1}$ may be more correct) and ranges from 160 to 220, whereas ergometer cycling EE can be additionally expressed as the percentage ratio of external work to energy expenditure (i.e., efficiency) with values ranging from 19% to 25% at submaximal exercise intensities. Individual variation in EE seems multifactorial (Williams & Cavanagh 1987) including both physiological and biomechanical variables. The aim of this study was to evaluate the importance of biochemical, structural physiological, anthropometric, and biomechanical influences on EE during cycle ergometer exercise and treadmill running.

In vitro studies have provided evidence that fast-twitch (FT) muscle fibres are less economical than slow-twitch (ST) muscle fibres (Jackman & Willis 1996) and this has also been demonstrated experimentally in human (Krustrup et al. 2008). It has furthermore been speculated that the high mitochondrial volume density ($Mito_{VD}$) of type-1 fibres is part of the reason for this fibre type being more economical (Jackman & Willis 1996). Initial studies associated determinants of cycling EE with a high prevalence of type-1 skeletal muscle fibres (Coyle et al. 1992; Mogensen et al. 2006), but this could not be confirmed in a more recent study including 40 volunteers (Hopker et al. 2013). In line with this the activity of skeletal muscle enzymes used as markers for $Mito_{VD}$ demonstrates no correlation to EE (Mogensen, Bagger 2006). Yet, on the other hand mitochondrial uncoupling protein 3 (UCP3) demonstrates a negative correlation to EE (Mogensen, Bagger 2006). This incoherence could be caused by damages to the mitochondrial membrane properties and function during the isolation preparation (Picard et al. 2011). Thus, to what extend skeletal muscle properties play a role for cycling EE remains an unresolved question. One aim with the current study was hence to investigate in greater detail the potential association between EE, skeletal muscle fibre type distribution, $Mito_{VD}$, selected mitochondrial proteins, and intrinsic mitochondrial function of intact permeabilised fibres. While cycling EE may correlate to properties of human skeletal muscle (Coyle, Sidossis 1992; Mogensen, Bagger

2006), these variables correlate even less to running EE (Bosco et al. 1987). Since running involves a less fixed movement pattern than cycling, more variables may be involved in the determinants of running than cycling EE.

During running, EE is amongst others determined by the time of force application, i.e. short ground contact time correlates with a higher mass-specific EE (Hoyt et al. 1994; Kram & Taylor 1990; Weyand et al. 2001). However, when stride length is manipulated to be short (and hence also increasing foot contact time), EE deteriorates (Cavanagh & Williams 1982), and likely illustrates the complex interplay between biomechanical variables. Vertical oscillation in the centre of mass (Tartaruga et al. 2012), low leg stiffness and the horizontal distance from the medial and lateral malleolus to the Achilles tendon (Barnes et al. 2014) are also related to running EE. To separate biomechanical determinants (as determined by three dimensional kinematic analysis) of running EE, we contrasted individual running and cycling EE considering that during cycle ergometer exercise the biomechanical influence on EE would be small because of the fixed movement pattern and therefore possible rank differences between running and cycling would be related to biomechanical factors since the biochemical, structural and physiological properties of the skeletal muscle are similar. It should nonetheless be realized that running likely includes greater contributions of isometric and lengthening contractions than does cycling and that the stretch-shortening cycle is thus likely more important in running.

With the aim to determine factors explaining variations in ergometer cycle and running EE 22 healthy male volunteers ranging from active to elite runners completed a series of comprehensive biochemical, histochemical, physiological, and biomechanical measures. For the purpose of this manuscript, we use the term EE when referring to steady state VO_2 or the percentage ratio of external work to energy expenditure during submaximal exercise.

Methods

The experimental protocols were approved by the local ethical committee (Dnr 1015-13, Gothenburg, Sweden) and conducted in accordance with the Declaration of Helsinki. Participants were fully informed about the purpose, benefit and risks associated with this study and provided their written and oral informed consent prior to initiation of the experiments.

Twenty-two healthy males (mean ± SD: age, 31.6 ± 7.8 yrs.; height, 183.3 ± 6.3 cm; body mass, 79.4 ± 13.3 kg) with a VO_2max of 60.5 ± 7.3 $ml.min^{-1}.kg^{-1}$ (range 45.5 to 72.1 $ml.min^{-1}.kg^{-1}$; VO_2max in $^{-1}$ 4.7 ± 0.6 $l.min^{-1}$; range 3.9 to 6.4 $l.min^{-1}$) participated in the study. The subjects ranged from being active (minimum running 30 km/week for last two years) to elite runners training more than 150 km/week. All participants were familiar with bicycling for commuting or leisure purposes but did not participate in bicycle training or competitions.

Experimental Design

The subjects reported to the laboratory over one month 1) to have a muscle biopsy taken and to determine body composition, 2) gas exchange variables during ergometer cycling and treadmill running, and 3) treadmill running to evaluate biomechanical measures

Skeletal Muscle Sampling

Skeletal muscle biopsies were obtained under standardized conditions from the *m. vastus lateralis* with the use of local anaesthesia (1 % lidocaine) using the Bergström technique with a needle modified for suction. The biopsy was divided into smaller parts and processed as follows: 20 mg tissue was placed in ice cold biopsy preservation solution medium for immediate respirometic analysis. Four 1 × 1 × 1 mm pieces were fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer for transmission electron microscopy (TEM). A part was snap frozen in liquid nitrogen for later preparation of skeletal muscle lysates for western blotting. Finally, a part was mounted in embedding medium (Tissue-Tek®, Sakura), frozen in isopentane cooled with liquid nitrogen for cryosectioning and immunohistochemistry.

Body Composition

Body weight (BW) in underwear was determined to the nearest 0.1 kg (Kern MPB300K100; Balingen, Germany) and height was determined to the nearest 0.5 cm. Lower leg mass was determined as described previously (Belanger et al. 1998; Lennihan & Mackereth 1973).

Exercise

Cycle economy. All cycling exercise was carried out on an ergometer (SRM, Jülich, Welldorf, Germany) with a 2 min warm-up at 20 W followed by 7 min at 150 W. Heart rate was recorded continuously, and VO_2 and carbon dioxide production (VCO_2) were determined in the 9^{th} min using the Douglas bag technique. After 20 min of rest, the procedure was repeated to obtain duplicate measures, and these data were subsequently averaged for data analysis. The bike ergometer was calibrated before each test according to the manufacturer's procedure (SRM, Jülich, Welldorf, Germany). Just before the start of the present study the power-meter was sent to the manufacturer for calibration and service.

Running economy. A treadmill (Rodby AS, Sweden) was used to determine running EE and VO_{2max}. 60 min after the cycle test, the subjects performed a warm-up including a 4 min run at a speed corresponding to approximately 50% of VO_{2max}. Following the warm-up, the subjects ran 3 times for 5 min at speeds corresponding to approximately 65%, 75% and 85% of VO_{2max}. At each speed of running, biomechanical recordings were obtained during minutes 2-3, and expired air was collected in Douglas bags during minutes 4-5 in order to determine running EE. In contrast to the experiments regarding cycling EE, running EE was not determined in duplicates. The treadmill speed was controlled using the length of the treadmill belt and measure the time of 10 turns with a stopwatch.

VO_{2max} test. The running economy test was followed by a VO_{2max} test. After 2-3 min rest a progressive running regimen that led to exhaustion within 5-8 min was performed. One min of running at an intensity sufficient to elicit VO_{2max} was followed by increments in inclination of 1% every min until the subject could no longer keep the pace. A levelling off in oxygen consumption and/or an RER-value of more than 1.10 was used as criterion for obtaining the VO_{2max} value.

Douglas bag procedure

Expired air was continuously collected in Douglas bags (C. Fritze Consulting, Svedala, Sweden) and later analysed for O_2, CO_2 and volume. The bags were flushed with expired air before they were used for the first time of the day. The fractional concentrations of oxygen were determined with a S-3A Oxygen analyser, and carbon dioxide concentrations were determined with the CD 3-A Carbon dioxide analyser, with a P-61B infrared sensor (AEI Technologies Inc., Naperville, IL, USA). The gas analysers were carefully checked beforehand for accuracy and linearity using high-precision gas mixture from one gas cylinder of 15.01% O_2 and 5.00% CO_2 and another gas cylinder with 21.00% O_2 and 0.03% CO_2 (Air Liquide AB, Kungsängen, Sweden). Expired gas volume was measured with a 90 L Tissot spirometer with a fast-responding temperature sensor attached on top of the inner cylinder. Since the volume often exceeded 90 L, the Douglas bags were in most cases analysed for volume in two stages. This approach has been verified previously (Rosdahl et al. 2010). Ambient room conditions were measured (accuracy: atmospheric pressure 0.2%; relative humidity 2%, GMH 3330, Griesinger electronic GmbH, Regenstauf, Germany).

Cycling EE was determined by steady state VO_2 ($l \cdot min^{-1}$) at 150 W. Moreover, energy expenditure during cycling was estimated through the respiratory exchange ratio (Lusk 1928) and thereby cycling efficiency (EE%) calculated by energy production/expenditure ratio expressed as a percentage. Running EE was determined by the ratio of VO_2 ($ml \cdot min^{-1} \cdot kg^{-1}$) and speed ($km \cdot h^{-1} \cdot 60^{-1}$). Also, energy expenditure during running was estimated by the respiratory exchange ratio and expressed as $kcal \cdot min^{-1} \cdot kg^{-1}$. In addition, running EE was computed without including body weight in the calculation, thus expressed in ml $O_2 \cdot km^{-1}$ units. The average running EE from the 3 running speeds aforementioned was taken as the outcome measure.

Three dimensional kinematics

A 14 camera infrared system (7 × Oqus3 and 7 × Oqus4 cameras, Qualisys, Gothenburg, Sweden) was used to capture each subject's pelvis and lower leg kinematics while running on the

treadmill. 34 reflecting markers were placed on each subject marking pelvis and both lower extremities. Temporal information about stance phase (SP) and flight phase (FP) was detected by defining touchdown events manually and toe-off events using an algorithm. Sagital joint motions of the hip, knee and ankle joints were calculated relative to the neutral standing position for 20 consecutive strides according to (Grood & Suntay 1983; Söderkvist & Wedin 1993). Here, the distal segment is rotating around the proximal segment with the first rotation about the sagittal x-axis, followed by the frontal y-axis and the transversal z-axis. Absolute and relative vertical displacement of the pelvis during loading phase (VDL_{ABS} and VDL_{REL}) and during push-off and flight phase (VDP_{ABS} and VDP_{REL}) was determined. Relative vertical displacement describes the vertical movement of the pelvis with regard to the standing reference value whereas the absolute values display the pelvis´absolute vertical displacement. Lastly, sagittal ankle path (AP) was calculated for the whole stride duration. For a complete list of discrete kinematic variables, see *Abbreviations*.

Transmission Electron Microscopy

The glutaraldehyde fixed biopsies were processed according to standard EM protocols, in short: the samples were block stained with 1% osmium tetraoxid and 1% uranyl acetate, then dehydrated and embedded in epon. After ultrathin sectioning the grids were post stained with Reynolds lead citrate. TEM image acquisition and stereological analysis were carried out as described in (Montero et al. 2015). A total of 4449 micrographs were analysed. For each subject 185 ± 17; 143 – 206 (mean ± SD; range), images were analysed for volume density of intermyofibrillar mitochondria (5.8 ±1.2 %; 3.2 - 7.8), of subsarcolemmal mitochondria (1.2 ± 0.49 %; 0.35 - 2.2) and of lipid droplets (0.4 ± 0.48 %; 0.03 - 2.05)

Cryosections

Tissue-Tek® embedded muscle samples were cut at -22˚C (Leica CM 1850, Leica Biosystems, Germany). Three serial transverse sections (8 µm) were obtained from each muscle sample and

mounted on glass slides (Thermo Scientific, Superfrost® Plus). The sections were set to air dry and stored at -20^{o} C until further processing

Skeletal Muscle Fibre Cross Sectional Area (FCSA)

Three serial transverse sections from each subject were fixed in acetone for 30 s and left for air drying at a room temperature for 10 min, and thereafter blocked with 5% goat serum in PBS. The sarcolemma of the muscle fibre was stained using anti-laminin mouse monoclonal primary antibody (1:80 dilution, NCL-Laminin, Novocastra). Alexa Flour 488 (1:600 dilution, ab150117, Abcam) was used as secondary antibody. Antibody incubations were performed at 37˚ C for 30 min in a humidified chamber. Sections were mounted with cover slips using a mounting medium (Vectashield® H-1000). All muscle sections were digitally captured at 10 × magnification on a Leica fluorescence microscopy (Leica DM500 B, Leica Microsystems, Germany). Semi-automated image processing and computation of FCSA was performed using ilastik (vers. 1.1.5;(Sommer et al. 2011)) for automated simple segmentation of the images and the FIJI software (NIH, USA) for determination of the FCSA using a custom-made plug-in that analysed the areas obtained from the simple segmentation as intensity maps. The FCSA was determined for 259 ± 100; 111 - 467 (mean ± SD; range) fibres per sample with an average FCSA of 5250 ± 1000 μm^2; 3507- 6963.

Muscle Capillarization

Three serial transverse sections from each subject were used for immunohistochemical analysis of capillary density as described in (Montero, Cathomen 2015). This density was determined by counting the number of capillaries surrounding coherent fibres and is expressed as the capillary-to-fibre ratio (C/F ratio). For each subject 186 ± 60; 118 - 375 (mean ± SD; range) capillaries surrounding 70 ± 29; 50 - 189, coherent fibres were counted. The C/F ratio was 2.20 ± 0.44; 1.41 - 2.96 (number · $fibre^{-1}$).

Fibre Typing

The skeletal muscle fibre types were assessed by the ATPase assay on three serial transverse sections from each subject as described previously (Montero, Cathomen 2015). The relative occurrence of fibre types for each subject was determined from a mean of 262 ± 63; 177-471 (mean ± SD; range fibers). The overall %-distribution was: ST: 63 ± 11; 45 - 86. FTa: 26 ± 8.8; 13- 47. FTx: 10 ± 8.8; 1.0 - 35.

Muscle Lysate Preparation and Western Blotting.

Snap frozen muscle sections (15-20 mg) were freeze dried for 15 hours at -55^{o}C (ScanVac CoolSafe55-4, Denmark), homogenized (Precellys 24 Tissue Homogenizer, Bertin Technologies, France) in freshly made homogenization buffer, after which muscle lysates were prepared (Nordsborg et al. 2012). Total protein concentrations were determined by BCA assay (Pierce, USA). Samples were diluted to a protein concentration of 2 µg per µl with 4 × Laemmli buffer and stored at -80^{o} C until analysis. Standard western blotting procedures were applied for quantification of mitofusin 2 (MFN2), uncoupling protein 3 (UCP3) and skeletal muscle specific alpha-actin (actin), in short: 30 µg of muscle lysate were separated on 12% polyacrylamide gels. For MFN2, the proteins were subsequently transferred onto PVDF membranes. Immunodetection was performed using the anti-MFN2 antibody (1:500 dilution, H00009927-M03, Abnova) and anti-alpha actin antibody (1:5000 dilution, A2066, Sigma-Aldrich). Anti-mouse or anti-rabbit IgG HRP-conjugated antibodies (W4021 and W4011, Promega) were used for labelling of primary antibodies. Labelled proteins were detected with Immobilon Western Chemiluminescent HRP Substrate (Merck-Millipore) using the Las-4000 image analyser system (Fujifilm Life Science). Quantification of band intensity was performed using FIJI software (NIH, USA). For UCP3, proteins were subsequently transferred onto nitrocellulose membranes and blotting efficiency was visualized by Ponceau S staining. Immunodetection was performed using the anti-UCP3 antibody (1:500 dilution, AB3046, MERCK Millipore) and a secondary anti-rabbit IgG HRP-conjugated antibody (1:5000 dilution, AP187, MERCK Millipore). Tagged proteins were detected with chemoluminescence (Femto kit; Pierce, Fisher Scientific, Wohlen, Switzerland) using the Chemidoc system with Quantity One software (Bio-Rad, Hercules, CA, USA) (Flück et al. 2014). The quantification of both MFN2 and UCP3 were normalized to actin.

Skeletal Muscle Mitochondrial Respiration

Immediately after a biopsy was taken, the muscle sample was processed as previously described in detail (Jacobs et al. 2012). Respiration measurements were carried out using the high-resolution Oxygraph-2k (Oroboros, Innsbruck, Austria) under hyperoxygenated conditions (200 to 450 nmol·O2 per ml) and at a temperature of 37°C by applying a SUIT (substrate, uncoupler, inhibitor titration) protocol (Jacobs, Siebenmann 2012). In the present study we were particularly interested in the following respiratory states: Leak respiration in absence of adenylates (L_N), induced with the addition of malate (2 mM) and octanoylcarnitine (0.2 mM). Fatty acid oxidation (FAO) was determined following the addition of saturating ADP (5 mM). Maximal oxidative phosphorylation (OXPHOS) capacity was reached by additional addition of pyruvate (5 mM), glutamate (10 mM) and succinate (20 mM). L_{omy} state was archived by titration of oligomycin (2.5µM), an ATP synthase inhibitor. The electron transport system (ETS) capacity was determinated with the titration of the proton ionophore, FCCP (carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone; 1-2.5 µM). Finally, cytochrome c oxidase (COX) activity was assessed by an initial inhibition of complex 3 with antimysin A (2.5 µM) followed by simultaneous titration of ascorbate (2 mM) and TMPD (0.5 mM) which are redox substrates that donate electrons directly to COX. Analysis of the respirometric experiments was carried out as described in (Pesta & Gnaiger 2012). COX activity was detected at an oxygen concentration of 285 µM, after subtraction of background activity, defined as the O_2 consumption in the presence of substrates but the absence of biological sample (Pesta & Gnaiger 2012). Mitochondrial efficiency can be indicated from coupling control ratios (CCR) (Jacobs, Siebenmann 2012; Pesta et al. 2011). FAO coupling control is the ratio between leak respiration without adenylates (L_N) and maximal fatty acid oxidative capacity (P_{FAO}); OXPHOS control ratio (phosphorylation system control ratio) is the ratio of OXPHOS/ETS and is an expression of the limitation of OXPHOS capacity by the phosphorylation system. The leak control ratio (LCR) is the ratio between L_{omy} and OXPHOS. Coupling control ratios are ratios of oxygen flux at a specific and constant mitochondrial substrate state. Mitochondrial respiratory capacity was assessed in two ways, by normalizing OXPHOS to COX activity (Jacobs, Siebenmann 2012) and by normalizing OXPHOS to $Mito_{VD}$ (Montero, Cathomen 2015).

Statistical analysis

Statistical analyses were performed using the IBM SPSS v. 20 (Chicago, USA) software package. Data were tested for normal distribution with the Kolmogorov-Smirnov test and for homogeneity of variances with Levene's test. To ensure that the ratios generated prior to statistical analysis, the slope of the relationship between logarithmically-transformed numerator and denominator were calculated to ensure there was no substantial deviation from 1 (1 fell within the 95% CI's of the slope) (Atkinson & Batterham 2012). Linear regression analyses were performed to determine association among variables. In all analyses, measures of cycling and running EE were considered as dependent variables. Pearson product-moment correlation coefficient was used to indicate the magnitude and direction of (univariate) associations with/without inclusion of BW or lower leg mass as covariates (i.e., with/without adjusting for BW or leg mass). Multiple regression analyses were used to identify variables independently associated with EE. Variables significantly associated with EE in univariate analyses were entered into the regression model as independent variables. In case of high correlation between independent variables, each variable was separately entered into the regression model to avoid high multicollinearity (variance inflation factor (VIF) > 10). In addition, association between cycling and running EE was assessed by Pearson product-moment correlation coefficient. A two-tailed *P*-value less than 0.05 was considered statistically significant.

Results

Cycling EE (VO_2 at 150 W).

No skeletal muscle parameter that in the past has been statistically associated with cycling EE such as structural features (number of capillaries per fibre and % slow twitch fibre distribution), mitochondrial properties (volume density, oxidative capacity, or mitochondrial efficiency determined as L_{OMY}/OXPHOS)) or protein content (UCP3 expression) could explain variation in cycling EE among subjects. Their correlations are shown in Figure 1.Also mitochondrial lipid droplet density (0.48 ± 0.48; 0.03 – 2.05 (mean ± SD; range), MFN2 protein expression (1.2 ± 0.4; 0.7 – 2.1), mitochondrial respiratory states such as L_N (14.2 ± 3.4; 8.3 - 21.1), FAO (30.8 ±

10.4; 14.9 – 52.1) and ETS (123.4 ± 31.2; 71.8 – 195.0), and mitochondrial coupling ratios such as L_N/P_{FAO} (0.51 ± 0.14; 0.24 - 0.74) and OXPHOS/ETS (0.89 ± 0.07; 0.76-1.00) had no associations to variations in cycling EE, which also held true for cycling EE% and running EE. Cycling EE was related to ventilation ($R^2 = 0.45$, $P = 0.03$) and anthropometrical variables such as BW ($R^2 = 0.23$, $P = 0.02$) and BMI ($R^2 = 0.23$, $P = 0.02$). In multiple regression analyses none of the aforementioned variables independently predicted cycling EE.

When BW was included as a covariate, no variable was correlated with cycling EE. Similar results were obtained with leg mass as a covariate.

*Cycling EE% (*cycling gross efficiency; energy production (at 150 W expressed in kcal) divided by energy expenditure (also in kcal)).

Cycling EE% was correlated with ventilation ($R^2 = 0.37$, $P < 0.01$), BW ($R^2 = 0.20$, $P = 0.03$) and BMI ($R^2 = 0.22$, $P = 0.03$) in univariate analyses. Only ventilation was independently associated with cycling EE ($\beta = -0.12$, partial $R^2 = 0.30$, $P = 0.01$) in multiple regression analyses.

With BW as a covariate, cycling EE was correlated with mitochondrial capacity given as OXPHOS/Total $Mito_{VD}$ ($R^2 = 0.37$, $P = 0.04$) and negatively correlated with ventilation ($R^2 = 0.34$, $P = 0.049$). In multiple regression analyses adjusted for BW, none of these variables were independently associated with cycling EE. Similar results were observed with leg mass as a covariate.

*Running EE (*average ml $O_2 \cdot kg^{-1} \cdot km^{-1}$ for 3 running speeds*).*

Whether expressing running EE in ml $O_2 \cdot kg^{-1} \cdot km^{-1}$ (Figure 1A) or in $kcal.kg^{-1}.min^{-1}$ (Figure 1B) the analytical outcome was similar. Biomechanical variables including VDL_{REL} ($R^2 = 0.18$, $P = 0.04$) and VDP_{REL} ($R^2 = 0.18$, $P = 0.04$) were correlated to running EE, while HFL_{INIT} ($R^2 = 0.35$, $P = 0.01$), HEX_{ROM} ($R^2 = 0.40$, $P = 0.01$), and AP ($R^2 = 0.24$, $P = 0.04$) were negatively correlated to running EE (ml · kg^{-1} · km^{-1}) in univariate analyses. Moreover, IMF $Mito_{VD}$ ($R^2 = 0.24$, $P = 0.02$) was correlated to running EE (ml · kg^{-1} · km^{-1}), but none of the other parameters determined in the muscle tissue correlated with running EE. In multiple regression analyses, AP ($\beta = -0.28$, partial $R^2 = 0.41$, $P = 0.02$) and IMF $Mito_{VD}$ ($\beta = 4.59$, partial $R^2 = 0.40$, $P = 0.02$) were independently associated with running EE (ml · kg^{-1} · km^{-1}) (Table 1).

With BW as a covariate, running EE (ml · km^{-1}) was correlated to VDL_{REL} ($R^2 = 0.67$, $P < 0.01$) and VDP_{REL} ($R^2 = 0.74$, $P < 0.01$) and negatively correlated to KFL_{INIT} ($R^2 = 0.35$, $P = 0.046$), KFL_{MAX} ($R^2 = 0.56$, $P < 0.01$), HFL_{INIT} ($R^2 = 0.48$, $P = 0.01$), HEX_{ROM} ($R^2 = 0.56$, $P < 0.01$) and ADF ($R^2 = 0.37$, $P = 0.04$). Moreover, running EE (ml · km^{-1}) was related to total $Mito_{VD}$ ($R^2 = 0.59$, $P < 0.01$), IMF $Mito_{VD}$ ($R^2 = 0.66$, $P < 0.01$), and the coupling control ratio, OXPHOS/ETS ($R^2 = 0.46$, $P = 0.02$) and negatively correlated with mitochondrial capacity when determined as OXPHOS/COX ($R^2 = 0.21$, $P = 0.03$). In multiple regression analyses adjusted for BW, none of the variables were independently associated with running EE (ml · km^{-1}). Yet, BW and running EE (ml · km^{-1}) showed an exceptionally high correlation ($R^2 = 0.94$, $P < 0.01$; Figure 3C).

Association between cycling EE/EE% and running EE (Figure 3E).
Running EE (ml · km^{-1}) was related to cycling EE ($R^2 = 0.27$, $P = 0.01$) and to cycling EE% ($R^2 = 0.25$, $P = 0.02$). In turn, there was no correlation when considering running EE as ml · kg^{-1} · km^{-1} rather than ml · km^{-1}, or if including BW or leg mass as a covariate.

Discussion

This cross-sectional study including healthy young and middle-aged males investigated potential determinants of cycling and running exercise economy (EE) from a comprehensive array of biochemical, structural, physiological, anthropometric, and biomechanical variables. The findings were: 1) cycling EE, as expressed by VO_2 or efficiency, is associated with BW, BMI and ventilation; 2) running EE correlates with BW, lower leg mass and to a minor degree with $Mito_{VD}$ and a large set of biomechanical variables related to vertical displacement; and 3) none of the aforementioned variables other than BW is a consistent and independent determinant of cycling or running EE if BW is included as a covariate. These findings suggest that from all potential contributors to EE assessed in this study, BW is the primary determinant of cycling and running EE.

Elucidation of the determinants of EE has remained an appealing but unresolved topic for exercise physiologists(Gaesser & Brooks 1975). Factors predicting EE have been advocated, but no single study has assessed their independent contribution to EE. Nonetheless, skeletal muscle must play a role for EE and inhibition of slow twitch muscle fibres elevates muscle oxygen

uptake and energy turnover during submaximal exercise (Krustrup, Secher 2008). At least for cycling EE we would predict the impact of 'external' (biomechanical) factors to be small. Muscle characteristics reported to predict EE include the percentage of type 1 skeletal muscle fibres (Coyle, Sidossis 1992; Hansen & Sjogaard 2007; Horowitz et al. 1994; Mogensen, Bagger 2006) and UCP3 protein content (Mogensen, Bagger 2006). Likewise, $Mito_{VD}$ is suggested to explain EE (Saunders et al. 2004). In the present study, none of the aforementioned variables nor any other measured muscle property correlated with cycling EE and only $Mito_{VD}$ was related to running EE, however, this was not observed when adjusting for BW. The reasons for these negative findings are unclear. It could be that the inherent variability of muscle biopsies have limited statistical power (Elder et al. 1982). To diminish this variability, Coyle et al. performed multiple biopsies at different locations in the *m. vastus lateralis* and found close association between the averaged percentage of type 1 fibres and EE in trained cyclists (Coyle, Sidossis 1992; Horowitz, Sidossis 1994). In contrast, the largest study (n = 40), presumably possessing a high statistical power, did not find an association between the percentage of type 1 fibres and EE in subjects presenting with widely differing training status (Hopker, Coleman 2013), similar to the present study. Whilst speculative, the influence of fibre type composition on EE may not be revealed when training status and thereby functional adaptations in skeletal muscle (e.g., efficiency of motor unit recruitment) are not relatively uniform among subjects (Barstow et al. 2000; Mallory et al. 2002). In addition, large between-group differences in BW were not accounted for the association of UCP3 and EE (Mogensen, Bagger 2006). Collectively, there is no evidence for any independent impact of skeletal muscle mitochondrial respiratory capacities, intermyofibrilar or subsarcolemmal mitochondrial volume density, fibre type distribution, capillariy or UCP3 and MFN2 protein content on EE in the current study.

BW was the main determinant of cycling and running EE and explained 94% of the variance in running EE as expressed as $ml \cdot km^{-1}$. Considering the weight bearing characteristics of running, the rationale for an impact of BW on running EE is obvious. In support hereof early work by Cavagna (Cavagna et al. 1964) demonstrated that the work of moving the limbs comprises a substantial part of the metabolic cost of running. Furthermore adding a few grams to the feet during running induces an increase in the metabolic rate (Myers & Steudel 1985). It has also been suggested that slender legs and a low body mass of Kenyan runners may explain their good

running economies (Larsen & Sheel 2015). It is less clear how BW appears to be dominant in influencing cycling EE. An explanation could be the effect of basal metabolic rate (BMR) on EE. Given that BMR increases according to BW (Harris & Benedict 1918), EE is influenced by BMR. The extent of such influence is inversely proportional to work rate (Ettema & Loras 2009) that was moderate (150 W) for most subjects during the cycling EE test. Thus, whilst BMR was not quantified, its impact on cycling EE can be presumed. Nonetheless, more than 50% of the variability of cycling EE remains unexplained and other factors than BW and leg mass must contribute to determine cycling EE. For example, muscle fibre coordination and activation at a given workload may influence cycling EE (Barnes & A.E. 2015; Moritani & deVries 1979; Paavolainen et al. 1999; Sale 1988). In that regard, neuromuscular adaptations may explain the improvement in cycling EE with strength training, while structural skeletal muscle adaptations observed with endurance training such as increased muscle capillarisation and $Mito_{VD}$ may have little impact (Hoppeler et al. 1985; Montero & Lundby 2015).

The energy cost of running is considered in relation to structural, physiological, anthropometrical, and biomechanical factors (Barnes & A.E. 2015). Stride length is correlated to running EE (Barnes, McGuigan 2014; Chapman et al. 2012; Tartaruga, Brisswalter 2012) with less efficient runners using a long stride length accompanied by shorter ground contact leading to a higher mass-specific metabolic cost of running (Chapman, Laymon 2012). In contrast, in the present study the ankle path during running, which may increase with longer stride length, correlated with improved running EE. This could be attributed, at least in part, by the observed concurrent positive association between vertical displacement and improved running EE. Moreover, a high running EE is associated with high hip and knee flexion as well as range of motion of the hip, all plausibly contributing to greater vertical displacement. Cavagna et al. (Cavagna et al. 2005) reported that increased vertical displacement results in lower internal work to accelerate the lower limbs, thus decreasing oxygen demand and the metabolic cost of running. Also, lower limb stiffness could contribute to increased vertical displacement and reduce the metabolic demand of running (Barnes & A.E. 2015; Barnes, McGuigan 2014). Despite multiple associations between biomechanics and running EE, none were significant after taking BW into account.

In contrast to our hypothesis, individual EE was not different between ergometer running and cycling. When BW was not taking into account in the analysis, cycling EE was correlated to running EE as expressed as ml · km-1. Thus, differences in cycling and running exercise protocols, e.g., related to biomechanics and differences related to the stretch-shortening cycle, play no more than a secondary role in determining EE. Otherwise, the correlation between cycling and running EE is expected given that cycling and running EE were primarily determined by a common variable, BW, as aforementioned. Beyond the predominant impact of BW, it remains to be established whether any unexplored independent contributing factor to EE differs between exercise modalities.

Perspective: Since there was no evidence for any independent impact of functional and structural attributes from skeletal muscle variables on EE, and while at the same time BW was the main determinant of cycling and running EE this should be taken into account when designing strength training programs for elite athletes.

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Conflict of interest

None declared.

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Tables

Table 1. Multiple linear regressions with running EE as the dependent variable

Model	***β* (95 % CI)**	***P***	$r_{partial}$	***Adjusted* R^2**
Model 1				
HFL_{INIT} (°)	-0.864 (-1.818, 0.090)	0.072	-0.52	0.65
HEX_{ROM} (°)	0.575 (-0.552, 1.703)	0.285	0.32	
AP (cm)	-0.276 (-0.497, -0.055)	**0.019**	-0.64	
VDL_{REL} (cm)	1.504 (-3.309, 6.317)	0.506	0.20	
VDP_{REL} (cm)	-0.492 (-4.822, 3.839)	0.807	-0.08	
IMF $Mito_{VD}$ (%)	4.583 (0.821, 8.345)	**0.021**	0.63	

AP, sagittal ankle path; *β*, unstandardized regression coefficient with running EE (average ml $O_2 \cdot kg^{-1} \cdot km^{-1}$ for 3 running speeds) as outcome; CI, confidence interval; EE, exercise economy; HEX_{ROM}, hip extension range of motion; HFL_{INIT}, initial hip flexion; IMF $Mito_{VD}$, intermyofibrillar mitochondrial volume density; VDL_{REL}, relative vertical displacement during loading phase; VDP_{REL}, relative vertical displacement during push-off phase

Figures and legends

Figure 1. Cost of running expressed as A) ml $O_2.kg^{-1}.min^{-1}$ and B) $kcal.kg^{-1}.min^{-1}$.

Figure 2. Correlational analysis between Cycling EE (left y-axis; black circles) and Running EE (right y-axis; white circles) and skeletal muscle capillaries ($number.fiber^{-1}$; cycling: Y= 0.031x + 2.0; running: Y= 5.7x + 187), percent slow twitch fibres (ST; %; cycling: Y= -0.0022x +2.2; running: Y= -0.068x + 204), mitochondrial volume density ($Mito_{VD}$, %; cycling: Y= -0.012x + 2.2; running: Y = 2.3 + 184), Uncoupling protein 3 expression (UCP3 expression; au; cycling: Y = -0.23x + 2.1; running: Y= 0.90x + 199), oxidative phosphorylation capacity (OXPHOS; pmol $O_2 \cdot sec^{-1} \cdot mg\ ww^{-1}$; cycling: Y = -0.0013x + 2.2; running: Y = 0.057x + 193) and leak control ratio (cycling: Y = 0.39x + 1.9; running: Y = -22.2x + 210). None of the measured skeletal muscle variables had an impact on cycling and running EE.

Figure 3. Correlational analysis between Cycling EE and Running EE and body weight (kg) and leg mass (kg). The lower left-hand panel shows the correlation between Running and Cycling EE.